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(54) Title: VACCINES, IMMUNOTHERAPEUTICS AND METHODS OF USING THE SAME

(57) Abstract: Compositions for and methods of enhancing, suppressing or otherwise modulating immune responses are disclosed. Improved vaccines which include a nucleotide sequence that encodes VEGF and an immunomodulating protein, both operably linked to regulatory elements are disclosed. The improved vaccines include DNA vaccines, recombinant vaccines for delivering foreign antigen and live attenuated vaccines. Methods of immunizing individuals are disclosed. Compositions for and methods of treating individuals with autoimmune diseases are disclosed.

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VACCINES, IMMUNOTHERAPEUTICS AND METHODS OF USING THE SAME

FIELD OF THE INVENTION

The present invention relates to improved vaccines, improved methods for prophylactically and/or therapeutically immunizing individuals against immunogens, and to improved immunotherapeutic compositions and improved immunotherapy methods.

BACKGROUND OF THE INVENTION

Immunotherapy refers to modulating a persons immune responses to impart a desirable therapeutic effect. Immunotherapeutics refer to those compositions which, when administered to an individual, modulate the individual's immune system to decrease symptoms and causes of symptoms brought on by undesirable immune responses or to alleviate symptoms or eliminate/reduce causes of symptoms by increasing desirable immune responses.

In some cases, immunotherapy is part of a vaccination protocol in which the individual is administered a vaccine that results in the individual being exposed to an immunogen. In such cases, the immunotherapeutic increases the immune response and/or selectively enhances a portion of the immune response which is desirable to treat or prevent the particular condition, infection or disease.

In some cases, immunotherapeutics are delivered free of immunogens. In such cases, the immunotherapeutics are provided to modulate the immune system by either decreasing or suppressing immune responses, enhancing or increasing immune responses,

decreasing or suppressing a portion of immune system, enhancing or increasing a portion of the immune system or decreasing or suppressing immune responses, enhancing or increasing immune responses. In some cases, immunotherapeutics include antibodies which when administered *in vivo*, bind to proteins involved in modulating immune responses. The interaction between antibodies and such proteins results in the alteration of immune responses. If the protein is involved in autoimmune disease, the antibodies can inhibit its activity in that role and reduce or eliminate the symptoms or disease. In some cases, immunotherapeutics include molecules that induce apoptosis in immune cells or other target cells.

Vaccines are useful to immunize individuals against target antigens such as allergens, pathogen antigens or antigens associated with cells involved in human diseases. Antigens associated with cells involved in human diseases include cancer-associated tumor antigens and antigens associated with cells involved in autoimmune diseases.

In designing such vaccines, it has been recognized that vaccines which produce the target antigen in the cell of the vaccinated individual are effective in inducing the cellular arm of the immune system. Specifically, live attenuated vaccines, recombinant vaccines which use avirulent vectors and DNA vaccines all lead to the production of antigens in the cell of the vaccinated individual. The presence of these antigens in the cells results in the induction of the cellular arm of the immune system. On the other hand, subunit vaccines which comprise only proteins and killed or inactivated vaccines, which do induce a humoral response, do not induce good cellular immune responses.

A cellular immune response is often necessary to provide protection against pathogen infection and to provide effective immune-mediated therapy for treatment of pathogen infection, cancer or autoimmune diseases. Accordingly, vaccines which produce the target antigen in the cell of the vaccinated individual such as live attenuated vaccines and recombinant vaccines which use avirulent vectors and DNA vaccines are preferred.

While such vaccines are often effective to immunize individuals prophylactically or therapeutically against pathogen infection or human diseases, there is a need for improved vaccines. There is a need for compositions and methods which produce an enhanced immune response.

SUMMARY OF THE INVENTION

The present invention relates to compositions which comprise vascular endothelial growth factor (VEGF) and/or nucleic acids that encode the same, in conjunction with immunomodulating proteins and/or nucleic acid molecules that encode the same, which together synergistically enhance and/or modulate the immune response, as well as methods of using such proteins and nucleic acid molecules. The delivery of VEGF in conjunction with immunomodulating proteins is useful for immunotherapy as well as for enhancing or otherwise tailoring immune responses in conjunction with vaccine delivery.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the titer of antibodies directed to the HSV gD protein in the serum of mice after treatment with DNA vaccines. The "negative control" DNA vaccine comprised the pCDNA3 plasmid. The "pgD + pCDNA3" vaccine comprised the coding sequence for the gD HSV protein operably linked to regulatory regions in the pCDNA3 plasmid. The "pgD + GM-CSF" vaccine comprised the coding sequence for the gD HSV protein and the coding sequence for GM-CSF, both operably linked to regulatory regions in the pCDNA3 plasmid. The "pgD + VEGF" vaccine comprised the coding sequence for the gD HSV protein and the coding sequence for VEGF, both operably linked to regulatory regions in the pCDNA3 plasmid. The "pgD + GM-CSF + VEGF" vaccine comprised the coding sequence for the gD HSV protein, the coding sequence for GM-CSF and the coding sequence for VEGF, all operably linked to regulatory regions in the pCDNA3 plasmid. Ten μg of DNA was injected into each mouse at 0, 3 and 9 weeks. Blood was drawn at 0, 3, 6, 9, 12 and 15 weeks, and the titer of antibodies raised to gD was determined by ELISA.

Figures 2A and 2B depict data from CTL stimulation assays.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The invention arises from the discovery that vascular endothelial growth factor (VEGF) works synergistically with immunomodulating proteins to enhance and/or modulate the immune response. Accordingly, such proteins may be delivered as immunotherapeutics to enhance, suppress or otherwise modulate immune responses.

VEGF proteins or nucleic acid molecules that encode the protein may be included as components in vaccine compositions or in compositions for targeting selective elimination of cells. The invention includes methods to modulate the immune system, both in general and to enhance the adaptive immune response towards a particular antigen or treat immunity related diseases or disorders. The invention also includes compositions for practicing the methods of the invention.

VEGF is a growth factor that is known to have a diverse role in the inflamation response. Genetic constructs encoding various combinations of VEGF, the immunomodulating protein GM-CSF and the antigen gD protein, all operably linked to mouse regulatory regions, were administered to mouse. Surprisingly, when the VEGF+GM-CSF+gD construct was administered, the titer of antibodies in the mouse serum towards the gD protein (~75,000) was more than additive of the increase in titer over the gD construct (positive control) of the GM-CSF+gD construct and the VEGF+gD construct (Fig. 1). Additionally, this synergistic stimulation of the immune system was also observed when the stimulation of the immune system was assayed by CTL stimulation assay (Fig. 2B).

VEGF is part of a family of proteins with structural and functional similarities. VEGF proteins include, but are not limited to VEGF, placenta growth factor (P1GF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E. It is contemplated that VEGF proteins and related proteins from all mammalian species can be used effectively in the present invention, however, VEGF and related proteins from *Homo sapiens* are used in preferred embodiments, VEGF from *Homo sapiens* as the most preferred embodiment.

As used herein, the term "immunomodulating proteins" is meant to refer to proteins and nucleic acid molecule expression products according to the present invention which enhance and/or modulate the immune response.

Immunomodulating proteins include chemokines, adhesion molecules, cytokines, co-stimulatory molecules, growth factors, and receptor molecules.

Chemokines that are immunomodulating proteins include MIP-1α, MIP-1β, RANTES, IL-8 and MCP-1.

Adhesion molecules that are immunomodulating proteins include members of the selectin family, mucin-like molecules, members of the integrin family, and members

of the immunoglobulin superfamily. Members of the selectin family that are immunomodulating proteins include L-selectin, P-selectin and E-selectin.

Mucin-like molecules are ligands to members of the selectin family. Mucin-like molecules that are immunomodulating proteins include CD34, GlyCAM-1 and MadCAM-1.

Members of the integrin family that are immunomodulating proteins include LFA-1, VLA-1, Mac-1 and p150.95.

Members of the immunoglobulin superfamily that are immunomodulating proteins include PECAM, ICAMs, ICAM-1, ICAM-2, ICAM-3, CD2 and LFA-3.

Cytokines that are immunomodulating proteins include M-CSF, GM-CSF, G-CSF, CSF, IL-4, IL-2, IL-12, IL-15, and mutant forms of IL-18 which include a deletion of the first about 35 amino acid residues present on the pro-form of the protein but not the mature form.

Co-stimulatory molecules that are immunomodulating proteins include B7.1, B7.2, CD40 and CD40 ligand (CD40L).

Growth factors that are immunomodulating proteins include vascular growth factor, IL-7 and nerve growth factor for example.

Receptor molecules that are immunomodulating proteins include Fas "death gene" expression product, tumor necrosis factor TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2 and DR6.

Other molecules include Caspase-1 (ICE).

One aspect of the invention is a method to modulate the immune response of a patient by delivering to them VEGF and at least one immunomodulating protein. VEGF and an immunomodulating protein may be administered as proteins, and/or as genetic constructs that will transfect the cells of the patient and will express VEGF and/or the immunomodulating protein.

According to some embodiments of the invention, VEGF and at least one immunomodulating protein is delivered by administering the proteins themselves. These proteins may be administered simultaneously or separately.

According to some embodiments of the invention, VEGF protein and an immunomodulating protein are delivered by administering at least one nucleic acid molecule which, when taken up by a cell, is expressed to produce the VEGF and immunomodulating proteins. The nucleic acids comprise the coding sequence of VEGF operably linked to regulatory regions and the coding sequence of at least one immunomodulating protein operably linked to regulatory regions. The nucleic acid molecules may be administered together or separately.

According to some embodiments of the invention, VEGF protein and an immunomodulating protein are delivered by administering at least one nucleic acid molecule which, when taken up by a cell, is expressed to produce the VEGF and immunomodulating proteins. The nucleic acids comprise the coding sequence of VEGF operably linked to regulatory regions and the coding sequence of at least one immunomodulating protein operably linked to regulatory regions. The nucleic acid molecules may be administered together or separately.

According to some embodiments of the invention, the immunomodulating protein is delivered by administering both nucleic acid molecules and proteins. The proteins and nucleic acids may be administered together or separately.

The administration of VEGF in combination with the immunomodulating protein, as either nucleic acid molecules and/or proteins is useful to enhance the patient's immune system. The proteins and nucleic acids may be administered together or separately. In particularly preferred embodiments, the immunomodulating proteins are GM-CSF, IL-12, IL-15, IL-2, B7.1, B7.2, or CD40 ligand.

According to some embodiments of the invention, a VEGF protein and at least one immunomodulating protein, either as proteins and/or a nucleic acid molecule(s) encoding the proteins, are administered as components of or otherwise as a supplement to a vaccine composition. The vaccine may be either a subunit vaccine, a killed vaccine, a live attenuated vaccine, a cell vaccine, a recombinant vaccine or a nucleic acid or DNA vaccine. In the case of a live attenuated vaccine, a cell vaccine, a recombinant vaccine or a nucleic acid or DNA vaccine, the VEGF protein and/or the immunomodulating protein may be encoded by the nucleic acid molecules of these vaccines. In particularly preferred

embodiments, the immunomodulating proteins are GM-CSF, IL-12, IL-15, IL-2, B7.1, B7.2, or CD40 ligand.

The compositions and methods of the invention are useful to modulate the immune response of a patient to induce and enhance cytotoxic T cell (CTL) responses, and/or induce and enhance antibody responses, and/or induce and enhance T cell proliferation responses. Accordingly, the compositions and methods may be used to particular advantage in conjunction or as part of a vaccine against intracellular pathogens, or against cells associated with autoimmune disease or cancer. Similarly, the method may be used in conjunction with the administration of live attenuated vaccines, cell vaccines, recombinant vaccines, and nucleic acid/DNA vaccines. Alternatively, the method is useful as an immunotherapeutic method for patients suffering from cancer or intracellular infection. The method and compositions are additionally useful to modulate immune response to treat immunocompromised patients or patients with lymphoproliferative diseases.

The compositions and methods to modulate immune response may be used to advantage to induce and enhance antibody responses, particularly when administered in conjunction or as part of a vaccine against bacteria, other extracellular pathogens, or those viruses for which antibody responses are protective such as hepatitis B virus, and in particular with subunit vaccines. Accordingly, the compositions and methods of the invention are useful as immunotherapeutics which are administered to patients suffering from undesirable CTL immune responses. Such shifting of the patient's immune system reduces the pathology caused by the CTL response. Finally, the compositions and methods for inducing and enhancing antibody responses are useful when administered to immunocompromised patients. The use of either GM-CSF or a nucleic acid molecule encoding GM-CSF or both is particularly useful when a strong antibody response or helper T cell response is particularly desirable.

The compositions and methods of the invention may be used to induce and enhance T cell proliferation responses, and will be particularly useful when administered in conjunction or as part of vaccines. Accordingly, the methods and compositions that induce and enhance T cell proliferation responses are useful as immunotherapeutics and when administered to immunocompromised patients.

In another aspect of the invention, VEGF and at least one immunomodulating protein that is a death protein such as FasL is administered to a patient. In preferred embodiments, a patient is administered composition comprising at least one nucleic acid molecule comprising the coding sequence of VEGF and the coding sequence of at least one immunomodulating protein that is a death protein such as FasL, both operably linking to regulatory regions. When administered to a patient, the nucleic acid of the composition will transfect the patient's cells and express the VEGF and FasL proteins. In particularly preferred embodiments, FasL is displayed on the surface of the transfected cells. The FasL ligand will induce apoptosis in any cell that it encounters having the Fas receptor. Other proteins have a similar function to FasL and may be used in the present invention. Such FasL substitutes include, but are not limited to, TNF-α, TNF-β, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6, and Caspase ICE.

Compositions and methods comprising VEGF and death proteins such as FasL and or nucleic acid molecules encoding such protein can regulate activated lymphocytes, primarily the T-cells and B-cells, in a patient. These methods and compositions can be used as immunotherapeutics for patients with lymphoproliferative diseases as well as immunodeficiency diseases. These methods and compositions could additionally be used to create areas of immune privilege in a patient, to, for example, prevent the rejection of transplanted organs.

In another aspect of the invention, a patient is administered a composition comprising at least one nucleic acid molecule comprising the coding sequence of VEGF, the coding sequence of at least one immunomodulating protein that is a death protein, and a target cell-specific ligand, both operably linking to regulatory regions. When administered to a patient, the nucleic acid of the composition will transfect the patient's cells and express the VEGF, a death protein and a cell-specific ligand protein. The cell-specific ligand will bind to the target cell, and the death protein will induce apoptosis in that target cell. In particularly preferred embodiments, FasL and the cell-specific ligand are displayed on the surface of the transfected cells. The target cell in this aspect is any cell in a patient that expresses Fas. Cell-specific ligands of interest include, but are not limited to, ligands to cell

surface receptors including cell surface protein binding partners, and antibodies to cell surface proteins.

Compositions and methods comprising VEGF, FasL and a cell specific ligand which specifically binds to B-cells or T-cells are useful for regulating specific populations of B-cells and T-cells. These compositions and methods are useful therapeutics to regulate populations of autoreactive B-cells and T-cells, among others.

One aspect of the present invention is a DNA vaccine. This aspect relates to methods and compositions for introducing genetic material into the cells of an individual in order to induce immune responses against proteins and peptides which are encoded by the genetic material. Similar DNA vaccines are described in U.S. Patent Nos. 5,593,972, 5,739,118, 5,817,637, 5,830,876, 5,962,428, 5,981,505, 5,580,859, 5,703,055, 5,676,594, and the priority applications cited therein, which are each incorporated herein by reference. In addition to the delivery protocols described in those applications, alternative methods of delivering DNA are described in U.S. Patent Nos. 4,945,050 and 5,036,006, which are both incorporated herein by reference.

The genetic material of the DNA vaccine is expressed by the individual's cells and serves as an immunogenic target against which an immune response is elicited. The resulting immune response is broad based: in addition to a humoral immune response, both arms of the cellular immune response are elicited. The methods of the present invention are useful for conferring prophylactic and therapeutic immunity. Thus, a method of immunizing includes both methods of immunizing against immunogens and thus for example of protecting an individual from pathogen challenge, or occurrence or proliferation of specific cells as well as methods of treating an individual suffering from pathogen infection, hyperproliferative disease or autoimmune disease.

The methods comprise the step of administering to the tissue of said individual, a composition that comprises at least one nucleic acid molecule comprising a nucleotide sequence that encodes a desired peptide or protein antigen, a nucleic acid sequence that encodes a VEGF protein and at least one nucleotide sequence that encodes an immunomodulating protein, each operably linked to a regulatory region. The composition may comprise one species of nucleic acid comprising all the coding sequences, or multiple species of nucleic acids each comprising a different complement of the aforementioned

coding sequences. The nucleic acid molecule(s) may be provided in plasmid DNA, recombinant vectors or as part of the genetic material provided in an attenuated vaccine or cell vaccine. Alternatively, one or more of VEGF, the immunomodulating protein(s) and desired peptide/protein antigen may be provided as protein in place of, or in addition to, the corresponding coding sequence. In some embodiments, these compositions and methods are administered as part of a vaccine protocol.

According to some aspects of the present invention, these compositions and methods for DNA vaccines are used prophylactically and/or therapeutically to immunize an individual against a pathogen or abnormal, disease-related cell. In this aspect, the composition of the DNA vaccine comprises genetic material that encodes an antigen peptide or protein that shares at least an epitope with an immunogenic protein found on the pathogen or cells to be targeted.

As used herein, the term "target protein" is meant to refer to peptides and protein encoded by gene constructs of the present invention which act as target proteins for an immune response. The term "target protein" and "immunogen" are used interchangeably and refer to a protein against which an immune response can be elicited. The target protein is an immunogenic protein which shares at least an epitope with a protein from the pathogen or undesirable cell-type such as a cancer cell or a cell involved in autoimmune disease against which immunization is required. The immune response directed against the target protein will protect the individual against and treat the individual for the specific infection or disease with which the target protein is associated.

The present invention is useful to elicit broad immune responses against a target protein, i.e. proteins specifically associated with pathogens, allergens or the individual's own "abnormal" cells. The present invention is useful to immunize individuals against pathogenic agents and organisms such that an immune response against a pathogen protein provides protective immunity against the pathogen. The present invention is useful to combat hyperproliferative diseases and disorders such as cancer by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. The present invention is useful to combat autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition.

The use of either GM-CSF or a nucleic acid molecule encoding GM-CSF or both in the DNA vaccine of the invention is particularly preferred when a strong antibody response or helper T cell response is particularly desirable. One example is a vaccine against hepatitis B. Other examples include vaccines against extracellular pathogens and allergens. The administration of either GM-CSF or a nucleic acid molecule encoding GM-CSF or both in the DNA vaccine is also useful for vaccinated individuals identified as being immunocompromised.

Some aspects of the present invention are compositions comprising genetic constructs. A genetic construct comprising DNA or RNA that encodes a VEGF protein, at least one target protein and at least one immunomodulating protein is introduced into the cells of tissue of an individual where it is expressed, thus producing the target protein. The DNA or RNA sequences encoding the target protein and immunomodulating protein are linked to regulatory elements necessary for expression in the cells of the individual. These regulatory elements for DNA expression include a promoter and a polyadenylation signal. In addition, other elements, such as a Kozak region, may also be included in the genetic construct.

As used herein, the term "genetic construct" refers to the DNA or RNA molecules that comprise a nucleotide sequence which encodes at least one target protein and which includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the vaccinated individual, a nucleotide sequence which encodes the VEGF protein and which includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the vaccinated individual and/or a nucleotide sequence which encodes at least one immunomodulating protein and which includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the vaccinated individual. In some embodiments, expressible form sequences that encode the target protein, VEGF and the immunomodulating protein are found on the same nucleic acid molecule that is delivered to the individual. In some embodiments, the expressible form sequences that encode the target protein, VEGF and the immunomodulating protein(s) occur separately or together on

more than one species of nucleic acid molecule. In such cases, all species of nucleic acid molecule are delivered to the individual, either together or separately.

As used herein, the term "expressible form" refers to gene constructs which contain the necessary regulatory elements operably linked to a coding sequence that encodes a target protein or an immunomodulating protein, such that when present in the cell of the individual, the coding sequence will be expressed.

As used herein, the term "sharing an epitope" refers to proteins which comprise at least one epitope that is identical to or substantially similar to an epitope of another protein.

As used herein, the term "substantially similar epitope" is meant to refer to an epitope that has a structure which is not identical to an epitope of a protein but nonetheless invokes a cellular or humoral immune response which cross reacts to that protein. An enhanced immune response against the target protein results.

When taken up by a cell, the genetic construct(s) may remain present in the cell as a functioning extrachromosomal molecule and/or integrate into the cell's chromosomal DNA. DNA may be introduced into cells where it remains as separate genetic material in the form of a plasmid or plasmids. Alternatively, linear DNA which can integrate into the chromosome may be introduced into the cell. When introducing DNA into the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication. Gene constructs may remain part of the genetic material in attenuated live microorganisms or recombinant microbial vectors which live in cells. Gene constructs may be part of genomes of recombinant viral vaccines where the genetic material either integrates into the chromosome of the cell or remains extrachromosomal.

Genetic constructs include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon and a polyadenylation signal. In addition, enhancers are often required for gene expression of the sequence that encodes the target protein or the immunomodulating protein. It is necessary that these elements be operably linked to the sequence that encodes the

desired proteins and that the regulatory elements are operable in the individual to whom they are administered.

Initiation codons and stop codons are generally considered to be part of a nucleotide sequence that encodes the desired protein. However, it is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatine and human metalothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to bovine growth hormone polyadenylation signal, SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal which is in pCEP4 plasmid (Invitrogen, San Diego, CA), referred to as the SV40 polyadenylation signal, is used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, CA) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region

which produces high copy episomal replication without integration. In some embodiments, the cDNA encoding the immunomodulating protein is inserted into pCDNA3.

In some preferred embodiments related to immunization applications, nucleic acid molecule(s) are delivered which include nucleotide sequences that encode a target protein, VEGF, the immunomodulating protein and, additionally, genes for proteins which further enhance the immune response against such target proteins. Examples of such genes are those which encode other cytokines and lymphokines such as GM-CSF, α-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12.

An additional element may be added which serves as a target for cell destruction if it is desirable to eliminate cells receiving the genetic construct for any reason. A herpes thymidine kinase (tk) gene in an expressible form can be included in the genetic construct. The drug gangcyclovir can be administered to the individual and that drug will cause the selective killing of any cell producing tk, thus, providing, the means for the selective destruction of cells with the genetic construct.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the cells the construct is administered into. Moreover, codons may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce DNA constructs which are functional in the cells.

Routes of administration include, but are not limited to, intramuscular, intranasally, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraoccularly and oral as well as topically, transdermally, by inhalation or suppository or to mucosal tissue such as by lavage to vaginal, rectal, urethral, buccal and sublingual tissue. Preferred routes of administration include administration to mucosal tissue, intramuscular, intraperitoneal, intradermal and subcutaneous injection. Genetic constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns."

The pharmaceutical compositions according to the present invention comprise about 1 nanogram to about 2000 micrograms of DNA. In some preferred embodiments, pharmaceutical compositions according to the present invention comprise

about 5 nanograms to about 1000 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 10 nanograms to about 800 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 0.1 to about 500 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 1 to about 350 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 25 to about 250 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 100 to about 200 micrograms of DNA.

The pharmaceutical compositions according to the present invention are formulated according to the mode of administration to be used. In cases where pharmaceutical compositions are injectable pharmaceutical compositions, they are sterile, pyrogen free and particulate free. An isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vaso-constriction agent is added to the formulation.

In some embodiments, the nucleic acid molecule is delivered to the cells in conjunction with administration of a polynucleotide function enhancer or a genetic vaccine facilitator agent. Polynucleotide function enhancers are described in U.S. Serial Number 08/008,342 filed January 26, 1993, U.S. Serial Number 08/029,336 filed March 11, 1993, U.S. Serial Number 08/125,012 filed September 21, 1993, and International Application Serial Number PCT/US94/00899 filed January 26, 1994, which are each incorporated herein by reference. Genetic vaccine facilitator agents are described in U.S. Serial Number 08/221,579 filed April 1, 1994, which is incorporated herein by reference. The co-agents which are administered in conjunction with nucleic acid molecules may be administered as a mixture with the nucleic acid molecule or administered separately, simultaneously, before or after administration of nucleic acid molecules. In addition, other agents which may function as transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with a GVF include growth factors, cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12 as well

as fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicles such as squalene, and hyaluronic acid. In some embodiments, an immunomodulating protein may be used as a GVF.

Nucleic acid molecules which are delivered to cells according to the invention may serve as genetic templates for proteins that function as prophylactic and/or therapeutic immunizing agents. In preferred embodiments, the nucleic acid molecules comprise the necessary regulatory sequences for transcription and translation of the coding region in the cells of the animal.

The present invention may be used to immunize an individual against all pathogens such as viruses, prokaryote and pathogenic eukaryotic organisms such as unicellular pathogenic organisms and multicellular parasites. The present invention is particularly useful to immunize an individual against those pathogens which infect cells which are not encapsulated such as viruses, and prokaryotes such as gonorrhea, listeria and shigella. In addition, the present invention is also useful to immunize an individual against protozoan pathogens which include a stage in the life cycle where they are intracellular pathogens. As used herein, the term "intracellular pathogen" is meant to refer to a virus or pathogenic organism that, at least part of its reproductive or life cycle, exists within a host cell and therein produces or causes to be produced, pathogen proteins. Table 2 provides a listing of some of the viral families and genera for which vaccines according to the present invention can be made. DNA constructs that comprise DNA sequences which encode the peptides that comprise at least an epitope identical or substantially similar to an epitope displayed on a pathogen antigen such as those antigens listed on the tables are useful in vaccines. Moreover, the present invention is also useful to immunize an individual against other pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites such as those listed on Table 3.

In order to produce a genetic vaccine to protect against pathogen infection, genetic material which encodes immunogenic proteins against which a protective immune response can be mounted must be included in a genetic construct as the coding sequence for the target. Whether the pathogen infects intracellularly, for which the present invention is particularly useful, or extracellularly, it is unlikely that all pathogen antigens will elicit a

protective response. Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The genetic construct used in the genetic vaccine can include genetic material which encodes many pathogen antigens. For example, several viral genes may be included in a single construct thereby providing multiple targets.

Tables 2 and 3 include lists of some of the pathogenic agents and organisms for which genetic vaccines can be prepared to protect an individual from infection by them. In some preferred embodiments, the methods of immunizing an individual against a pathogen are directed against HIV, HTLV or HBV.

Another aspect of the present invention provides a method of conferring a broad based protective immune response against hyperproliferating cells that are characteristic in hyperproliferative diseases and to a method of treating individuals suffering from hyperproliferative diseases. As used herein, the term "hyperproliferative diseases" is meant to refer to those diseases and disorders characterized by hyperproliferation of cells. Examples of hyperproliferative diseases include all forms of cancer and psoriasis.

It has been discovered that introduction of a genetic construct that includes a nucleotide sequence which encodes an immunogenic "hyperproliferative cell"- associated protein into the cells of an individual results in the production of those proteins in the vaccinated cells of an individual. As used herein, the term "hyperproliferative-associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease. To immunize against hyperproliferative diseases, a genetic construct that includes a nucleotide sequence which encodes a protein that is associated with a hyperproliferative disease is administered to an individual.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides which comprise at least an epitope found on such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein which is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein. Such target proteins include those which

are proteins encoded by oncogenes such as *myb, myc, fyn*, and the translocation gene *bcr/abl, ras, src*, P53, *neu, trk* and EGRF. In addition to oncogene products as target antigens, target proteins for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell lymphomas which, in some embodiments, are also used as target antigens for autoimmune disease. Other tumor-associated proteins can be used as target proteins such as proteins which are found at higher levels in tumor cells including the protein recognized by monoclonal antibody 17-1A and folate binding proteins.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to prophylactically immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse. Developments in genetics and technology as well as epidemiology allow for the determination of probability and risk assessment for the development of cancer in an individual. Using genetic screening and/or family health histories, it is possible to predict the probability a particular individual has for developing any one of several types of cancer.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer or are otherwise in remission are particularly susceptible to relapse and reoccurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat a recurrence. Thus, once it is known that an individual has had a type of cancer and is at risk of a relapse, they can be immunized in order to prepare their immune system to combat any future appearance of the cancer.

The present invention provides a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of genetic constructs serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies.

T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease, and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include Vβ-3, Vβ-14, Vβ-17, and Vα-17. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA. See: Howell, M.D., *et al.*, 1991 *Proc. Natl. Acad. Sci. USA* **88**:10921-10925; Paliard, X., *et al.*, 1991 *Science* **253**:325-329; Williams, W.V., *et al.* 1992 *J. Clin. Invest.* **90**:326-333; each of which is incorporated herein by reference.

In MS, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-7 and Vα-10. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS. See: Wucherpfennig, K.W., *et al.*, 1990 *Science* **248**: 1016-1019; Oksenberg, J.R., *et al.*, 1990 *Nature* **345**:344-346; each of which is incorporated herein by reference.

In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-6, Vβ-8, Vβ-14 and Vα-16, Vα-3C, Vα-7, Vα-14, Vα-15, Vα-16, Vα-28 and Vα-12. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be

taken and the variable region of those TCRs identified using standard techniques. Genetic vaccines can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies which bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody.

In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Genetic vaccines can be prepared using this information.

In the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes DNA constructs that encode the variable region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, *et al.* 1987 *Sequence of Proteins of Immunological Interest* U.S. Department of Health and Human Services, Bethesda MD, which is incorporated herein by reference. In addition, a general method for cloning functional variable regions from antibodies can be found in Chaudhary, V.K., *et al.* 1990 *Proc. Natl. Acad. Sci.* USA 87: 1066, which is incorporated herein by reference.

In addition to using expressible forms of immunomodulating protein coding sequence to improve genetic vaccines, the present invention relates to improved attenuated live vaccines and improved vaccines which use recombinant vectors to deliver foreign genes that encode antigens. Examples of attenuated live vaccines and those using recombinant vectors to deliver foreign antigens are described in U.S. Patent Nos.: 4,722,848; 5,017,487;

5,077,044; 5,110,587; 5,112,749; 5,174,993; 5,223,424; 5,225,336; 5,240,703; 5,242,829; 5,294,441; 5,294,548; 5,310,668; 5,387,744; 5,389,368; 5,424,065; 5,451,499; 5,453,364; 5,462,734; 5,470,734; and 5,482,713, which are each incorporated herein by reference. Gene constructs are provided which include the nucleotide sequence that encodes an immunomodulating protein is operably linked to regulatory sequences that can function in the vaccinee to effect expression. The gene constructs are incorporated in the attenuated live vaccines and recombinant vaccines to produce improved vaccines according to the invention.

The present invention provides an improved method of immunizing individuals that comprises the step of delivering gene constructs to the cells of individuals as part of vaccine compositions which include DNA vaccines, attenuated live vaccines and recombinant vaccines. The gene constructs comprise a nucleotide sequence that encodes VEGF and at least one immunomodulating protein, each of which are operably linked to regulatory sequences that can function in the vaccinee to effect expression. The improved vaccines result in an enhanced cellular immune response.

Pharmaceutical compositions for treating autoimmune disease comprise an antibody specific for a chemokine and a pharmaceutically acceptable carrier. According to preferred embodiments, the compositions are injectable. The sterile, pyrogen-free, particulate-free injectable compositions comprise one or more an antibodies specific for a chemokine and a pharmaceutically acceptable carrier or injection vehicle.

EXAMPLE 1

Chemokines:

Some embodiments of the invention provide compositions comprising and the administration of VEGF and/or nucleic acid molecules that encode VEGF in combination with chemokines and/or nucleic acid molecules that encode chemokines.

MCP-1 is particularly useful in inducing and enhancing CD8+ CTLs.

MIP-1α is particularly useful in the induction of antibodies.

IL-8 is particularly useful in the induction of antibodies, and is a strong inducer of T helper responses.

RANTES induces TH1 as well as CTL responses.

MIP-1β, such as the construct which has been cloned into pCDNA3 to generate pCDNA3- MIP-1β, may also be used.

Adhesion molecules:

Some embodiments of the invention provide compositions comprising and the administration of VEGF and/or nucleic acid molecules that encode VEGF in combination with adhesion molecules and/or nucleic acid molecules that encode adhesion molecules.

Members of the selectin family of Adhesion molecules:

- L-selectin
- P-selectin
- E-selectin

Mucin-like Adhesion molecules:

- CD34
- GlyCAM-1 such as the construct which has been cloned into pCDNA-3 to generate pCDNA3-GlyCAM-1
- MadCAM-1

Members of the integrin family of Adhesion molecules:

- LFA-1
- VLA-1
- Mac-1
- p150.95

Adhesion molecule Members of the immunoglobulin superfamily:

- PECAM
- ICAMs
  - ICAM-1
  - ICAM-2
  - ICAM-3
- CD2
- LFA-3

Adhesion molecules are most useful when administered as nucleic acid molecules.

Adhesion molecules are most useful when administered as nucleic acid molecules as part of or in conjunction with vaccines, particularly live attenuated vaccines, cell vaccines, recombinant vaccines, and nucleic acid/DNA vaccines.

Adhesion molecules useful when delivered as nucleic acid molecules intratumor or intralesion.

Preferred adhesion molecules include ICAM-1, LFA-3 and E-selectin.

ICAM-1 is best for CTL and proliferation.

Cytokines:

Some embodiments of the invention provide compositions comprising and the administration of VEGF and/or nucleic acid molecules that encode VEGF in combination with cytokines and/or nucleic acid molecules that encode cytokines.

M-CSF

G-CSF

CSF

IL-4

mutant forms of IL-18

GM-CSF

IL-12

IL-15

IL-2

Co-stimulatory molecules:

Some embodiments of the invention provide compositions comprising and the administration of VEGF and/or nucleic acid molecules that encode VEGF in combination with co-stimulatory molecules and/or nucleic acid molecules that encode co-stimulatory molecules.

CD40 such as the construct in which cDNA encoding CD40 is cloned into pCDNA3 to generate pCDNA3-CD40 may be used.

CD40L

B7.1

B7.2

Growth factors:

Some embodiments of the invention provide compositions comprising and the administration of VEGF and/or nucleic acid molecules that encode VEGF in combination with growth factor molecules and/or nucleic acid molecules that encode growth factor molecules.

Vascular growth factor such as the construct in which cDNA encoding vascular growth factor is cloned into pCDNA3 to generate pCDNA3-VGF may be used.

IL-7

nerve growth factor

vascular endothelial growth factor (VEGF)

Receptor molecules/death proteins:

Some embodiments of the invention provide compositions comprising and the administration of VEGF and/or nucleic acid molecules that encode VEGF in combination with receptor molecules and/or nucleic acid molecules that encode receptor molecules.

Fas "death gene" expression product

TNF receptor

Flt

Apo-1

p55

WSL-1

DR3

TRAMP

Apo-3

AIR

LARD

NGRF

DR4

DR5

KILLER

TRAIL-R2

TRICK2

DR6

Caspase (ICE)

Table 1 lists the GENBANK Accession numbers and journal citations for the nucleotide and amino acid sequences for each of the above immunomodulating proteins.

Table 1

MadCAM-1	
Accession:	U80016
Authors:	Leung, E., et al.
Journal:	Immunogenetics 46 (2), 111-119 (1997)
MadCAM-1	
Accession:	U43628
Authors:	Shyjan, A.M., et al.
Journal:	J. Immunol. 156 (8), 2851-2857 (1996)
NGF	
Accession:	M57399
Authors:	Kretschmer, P.J., et al.
Journal:	Growth Factors 5, 99-114 (1991)
IL-7	
Accession:	J04156
Authors:	Goodwin, R.G., et al.
Journal:	Proc. Natl. Acad. Sci. U.S.A. 86 (1), 302-306 (1989)
VEGF	
Accession:	M32977
Authors:	Leung, D.W., et al.
Journal:	Science 246, 1306-1309 (1989)
TNF-R	
Accession:	M60275
Authors:	Gray, P.W., et al.
Journal:	Proc. Natl. Acad. Sci. U.S.A. 87, 7380-7384 (1990)
TNF-R	
Accession:	M63121
Authors:	Himmler, A., et al.
Journal:	DNA Cell Biol. 9, 705-715 (1990)
Fas	
Accession:	M67454
Authors:	Itoh, N., et al.
Journal:	Cell 66 (2), 233-243 (1991)
CD40L	
Accession:	L07414
Authors:	Gauchat, J.F.M., et al.
Journal:	FEBS Lett. 315, 259-266 (1992)

IL-4
Accession: M23442
Authors: Arai, N., et al.
Journal: J. Immunol. 142 (1), 274-282 (1989)

IL-4
Accession: M13982
Authors: Yokota, T., et al.
Journal: Proc. Natl. Acad. Sci. U.S.A. 83 (16), 5894-5898 (1986)

CSF
Accession: M37435
Authors: Wong, G.G., et al.
Journal: Science 235 (4795), 1504-1508 (1987)

G-CSF
Accession: X03656
Authors: Nagata, S., et al.
Journal: EMBO J. 5 (3), 575-581 (1986)

G-CSF
Accession: X03655
Authors: Nagata, S., et al.
Journal: EMBO J. 5 (3), 575-581 (1986)

GM-CSF
Accession: M11220
Authors: Lee, F., et al.
Journal: Proc. Natl. Acad. Sci. U.S.A. 82 (13), 4360-4364 (1985)

GM-CSF
Accession: M10663
Authors: Wong, G.G., et al.
Journal: Science 228 (4701), 810-815 (1985)

M-CSF
Accession: M27087
Authors: Takahashi, M., et al.
Journal: Biochem. Biophys. Res. Commun. 161 (2), 892-901 (1989)

M-CSF
Accession: M37435
Authors: Wong, G.G., et al.
Journal: Science 235 (4795), 1504-1508 (1987)

LFA-3
Accession: Y00636
Authors: Wallner, B.P., et al.
Journal: J. Exp. Med. 166 (4), 923-932 (1987)

ICAM-3
Accession: X69819
Authors: de Fougerolles, A.R., et al.
Journal: Unpublished

ICAM-2
Accession: X15606
Authors: Staunton, D.E., et al.
Journal: Nature 339 (6219), 61-64 (1989)

ICAM-1
Accession: J03132
Authors: Staunton, D.E., et al.
Journal: Cell 52 (6), 925-933 (1988)

PECAM
Accession: M28526
Authors: Newman, P.J., et al.
Journal: Science 247, 1219-1222 (1990)

p150.95
Accession: Y00093
Authors: Corbi, A.L., et al.
Journal: EMBO J. 6 (13), 4023-4028 (1987)

Mac-1
Accession: J03925
Authors: Corbi, A.L., et al.
Journal: J. Biol. Chem 263 (25), 12403-12411 (1988)

LFA-1
Accession: Y00796
Authors: Larson, R., et al.
Journal: J. Cell Biol. 108 (2), 703-712 (1989)

CD34
Accession: M81104
Authors: Simmons, D.L., et al.
Journal: J. Immunol. 148, 267-271 (1992)

RANTES
Accession: M21121
Authors: Schall, T.J., et al.
Journal: J. Immunol. 141, 1018-1025 (1988)

IL-8
Accession: M28130
Authors: Mukaida, N., et al.
Journal: J. Immunol. 143 (4), 1366-1371 (1989)

MIP-1α
Accession: U72395
Authors: Fridell, R.A., et al.
Journal: J. Cell. Sci. 110 (Pt 11), 1325-1331 (1997)

E-selectin
Accession: M24736
Authors: Bevilacqua, M.P., et al.
Journal: Science 243 (4895), 1160-1165 (1989)

CD2
Accession: M14362
Authors: Sewell, W.A., et al.
Journal-1: Proc. Natl. Acad. Sci. U.S.A. 83, 8718-8722 (1986)
Journal-2: Proc. Natl. Acad. Sci. U.S.A. 84, 7256 (1987)

CD2
Accession: M16336
Authors: Sayre, P.H., et al.
Journal: Proc. Natl. Acad. Sci. U.S.A. 84 (9), 2941-2945 (1987)

MCP-1
Accession: S69738
Authors: Li, Y.S., et al.
Journal: Mol. Cell. Biochem. 126 (1), 61-68 (1993)

MCP-1
Accession: S71513
Authors: Yoshimura, T., et al.
Journal: Adv. Exp. Med. Biol. 305, 47-56 (1991)

L-selectin
Accession: X16150
Authors: Tedder, T.F., et al.
Journal: J. Exp. Med. 170 (1), 123-133 (1989)

P-selectin
Accession: M25322
Authors: Johnston, G.I., et al.
Journal: Cell 56, 1033-1044 (1989)

FLT
Accession: X94263
Authors: Mandriota, S.J., et al.
Journal: J. Biol. Chem. 271 (19), 11500-11505 (1996)

FLT
Accession: X51602
Authors-1: Shibuya, M., et al.
Journal-1: Oncogene 5 (4), 519-524 (1990)
Authors-2: Han, H.J., et al.
Journal-2: Hum. Mol. Genet. 2 (12), 2204 (1993)

Apo-1
Accession: X63717
Authors: Oehm *et al.*,
Journal: J. Biol. Chem., **1992**, 267 (15), 10709-15

Fas
Accession: M67454
Authors: Itoh *et al.*,
Journal: Cell, **1991**, 66(2), 233-43

TNFR-1
Accession: M67454
Authors: Nophar *et al.*,
Journal: EMBO J., **1990**, 9 (10), 3269-78

p55
Accession: M58286
Authors: Loetscher *et al.*,
Journal: Cell, **1990**, 61, 351-359

WSL-1
Accession: Y09392
Authors: Kitson *et al.*,
Journal: Nature, **1996**, 384 (6607), 372-5

DR3
Accession: U72763
Authors: Chinnaiyan *et al.*,
Journal: Science, **1996**, 274 (5829), 990-2

TRAMP
Accession: U75381
Authors: Bodmer *et al.*
Journal: Immunity, **1997**, 6 (1), 79-88

Apo-3
Accession: U74611
Authors: Marsters *et al.*
Journal: Curr. Biol., **1996**, 6 (12), 1669-76

AIR
Accession: U78029
Authors: Degli-Esposti *et al.*,
Journal:

LARD
Accession: U94512
Authors: Screaton *et al.*,
Journal: Proc. Natl. Acad. Sci. U.S.A., **1997**, 94 (9), 4615-19

NGRF
Accession: M14764
Authors: Johnson *et al.*,
Journal: Cell, **1986**, 47 (4), 545-554

DR4 (TRAIL)
Accession: U90875
Authors: Pan *et al.*,
Journal: Science, **1997**, 276 (5309), 111-113

DR5
Accession: AF012535
Authors: Sheridan *et al.*,
Journal: Science, **1997**, 277 (5327), 818-821

KILLER
Accession:
Authors: Wu *et al.*,
Journal: Nature Genetics, **1997**, 17 (2): 141-143

TRAIL-R2
Accession: AF020501
Authors: MacFarlane *et al.*,
Journal: J. Biol. Chem., **1997**, 272 (41): 25417-25420

TRICK2
Accession: AF018657
Authors: Screaton *et al.*,
Journal: Curr. Biol., **1997**, 7 (9): 693-696.

DR6
Accession: AF068868
Authors: Pan *et al.*,
Journal:

ICE
Accession: U13698
Authors: Alnemri, E.S., et al.
Journal: J. Biol. Chem. 270 (9), 4312-4317 (1995)

ICE
Accession: U13697
Authors: Alnemri, E.S., et al.
Journal: J. Biol. Chem. 270 (9), 4312-4317 (1995)

ICE
Accession: U13699
Authors: Alnemri, E.S., et al.
Journal: J. Biol. Chem 270 (9), 4312-4317 (1995)

VLA-1
Accession: X17033
Authors: Takada., et al.
Journal: J. Biol. Chem. 109 (1), 397-407 (1989)

CD86 (B7.2)
Accession: U04343
Authors: Azuma, et al.
Journal: Nature. 366 (6450), 76 (1993)

CD40 ligand
Accession No. P29965
Graf, D. et al.
Eur. J. Immunol. 22 (12) 3191-3194 (1992)
Hollenbaugh, D. et al.
Embo. J. 11 (12) 4313-4321 (1992)
Spriggs, M.K. et al.
Cell 72 291-300 (1993)
Spriggs, M.K. et al.
J. Exp. Med. 176 (6) 1543-1550 (1992)
Gauchat et al.

Febs. Lett. 315 (3) 259-266 (1993)

CD86
Accession No. 5901920
Azuma et al.
Nature 366 (6450) 76-79 (1993)
Reeves et al.
Mamm. Genome 8 (8) 581-582 (1997)

CD80 (B7.1)
Accession No. 4885123
Selvakumar et al.
Immunogenetic 36 (3) 175-181 (1992)
Freeman et al.
Blood 79 (2) 489-494 (1992)

CD40
Accession No. 4507581
Stamenkovic et al.
Embo. J. 8 (5) 1403-1410 (1989)

IL-15

The nucleotide and amino acid sequences of human IL-15 are well known and set forth in Grabstein, et al. (1994) Science 264:965-968, and accession code Swissprot U03099, which are each incorporated herein by reference.

IL-2

The nucleotide and amino acid sequences of human IL-2 are well known and set forth in Holbrook, et al. (1984) Proc. Natl. Acad. Sci. USA 81:1634-1638, Fujita, et al. (1983) Proc. Natl. Acad. Sci. USA 80:7437-7441, Fuse, et al. (1984) Nucl. Acids Res. 12:9323-9331, Taniguchi, et al. (1983) Nature 302:305-310, Maeda, et al. (1983) Biochem. Biophys. Res. Comm. 115:1040-1047, Devos, et al. (1983) Nucl. Acids Res. 11:4307-4323, and accession code Swissprot PO1585, which are each incorporated herein by reference.

IL-12

IL-12 is described in published PCT application WO 90/05147 published May 17, 1990 which is incorporated herein by reference. Wolf, S.F. *et al.* 1991 *J. Immunol.* 146 (9):3074-3081, which is incorporated herein by reference, discloses the nucleotide

sequence of cDNA that encodes IL-12 as well as the predicted amino acid sequence of the IL-12 protein. Native human IL-12 protein consists of two subunits, p35 and p40. The two subunits form a heterodimeric complex that is biologically active.

According to some embodiments of the invention, the nucleotide sequences that encode each subunit of IL-12 are on a single plasmid, non-plasmid nucleic acid molecule, or viral or microbial genome, wherein the nucleotide sequence encoding each subunit being under the control of its own set of regulatory elements. In some preferred embodiments, coding sequences for both subunits of Il-12 are on a single plasmid; each coding sequence being operably linked to its own set of regulatory elements. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on the same plasmid as the coding sequences for both subunits. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on a separate plasmid from a plasmid which contains the coding sequences for both subunits and the two plasmids are delivered to an individual.

According to some embodiments of the invention, the nucleotide sequence that encodes the p35 subunit is on a first plasmid and the nucleotide sequence that encodes the p40 subunit is on a second plasmid and the two plasmids are co-administered to the same site on an individual. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on the same plasmid as the coding sequences for the p35 subunit. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on the same plasmid as the coding sequences for the p40 subunit. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on a separate plasmid from either plasmid which contains the coding sequences for respective subunits and the three plasmids are delivered to an individual.

IL-12 protein, and the nucleotide sequence encoding it, may be modified so that the two subunits are encoded by a single nucleotide sequence and expressed as a single chain (fusion) protein molecule. According to the invention, a linker amino acid sequence is provided which essentially connects the two subunits but which is flexible enough so that a biologically active protein can form by the complexing of different portions of the single chain protein. Figure 8A shows an example of a single chain protein in which the coding

sequence for the single chain protein is under the control of a human cytomegalovirus promoter. The coding sequence of the single chain protein includes, from 5' to 3', the coding sequence of the p35 subunit, a coding sequence for a linker and the coding sequence of the p40 subunit as a single coding sequence. It is contemplated that in an alternative arrangement, the coding sequence of the single chain protein includes the coding sequence of the p40 subunit, a coding sequence for a linker and the coding sequence of the p35 subunit as a single coding sequence. The linker must be long enough and flexible enough to allow the two parts of the single protein to assume positions relative to each other such that a biologically active complex is formed.

According to some embodiments of the invention, the nucleotide sequences that encode single chain IL-12 proteins in which the two subunits are joined by a linker to form a single protein are incorporated into a plasmid, non-plasmid nucleic acid molecule, or viral or microbial genome, and operably linked to regulatory elements necessary for expression in eukaryotic cells. In preferred embodiments, the nucleotide sequences that encode the single chain proteins in which the two subunits are joined by a linker to form a single protein are incorporated into a plasmid. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on the same plasmid as the coding sequences for the single chain IL-12 protein. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on a separate plasmid from the plasmid which contains the coding sequences for the single chain protein and the two plasmids are delivered to an individual.

Table 2

Picornavirus Family

Genera: Rhinoviruses: (Medical) responsible for ~50% cases of the common cold.
Enteroviruses: (Medical) includes polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus.
Apthoviruses: (Veterinary) these are the foot and mouth disease viruses.

Target antigens: VP1, VP2, VP3, VP4, VPG

Calcivirus Family

Genera: Norwalk Group of Viruses: (Medical) these viruses are an important causative agent of epidemic gastroenteritis.

Togavirus Family

Genera: Alphaviruses: (Medical and Veterinary) examples include Senilis viruses, RossRiver virus and Eastern & Western Equine encephalitis.
Reovirus: (Medical) Rubella virus.

Flariviridue Family

Examples include: (Medical) dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses.

Hepatitis C Virus: (Medical) these viruses are not placed in a family yet but are believed to be either a togavirus or a flavivirus. Most similarity is with togavirus family.

Coronavirus Family: (Medical and Veterinary)
Infectious bronchitis virus (poultry)
Porcine transmissible gastroenteric virus (pigs)
Porcine hemagglutinating encephalomyelitis virus (pigs)
Feline infectious peritonitis virus (cats)
Feline enteric coronavirus (cats)
Canine coronavirus (dogs)
The human respiratory coronaviruses cause ~40 cases of common cold. EX. 224E, 0C43
Note- coronaviruses may cause non-A, B or C hepatitis

Target antigens:
E1- also called M or matrix protein
E2- also called S or Spike protein
E3- also called HE or hemagglutin- elterose glycoprotein
(not present in all coronaviruses)

N - nucleocapsid

Rhabdovirus Family
Genera: Vesiliovirus
Lyssavirus: (Medical and Veterinary) rabies
Target antigen: G protein
N protein
Filoviridue Family: (Medical)
Hemorrhagic fever viruses such as Marburg and Ebola virus

Paramyxovirus Family
Genera: Paramyxovirus: (Medical and Veterinary)
Mumps virus, New Castle disease virus (important pathogen in chickens)
Morbillivirus: (Medical and Veterinary)
Measles, canine distemper
Pneuminvirus: (Medical and Veterinary)
Respiratory syncytial virus

Orthomyxovirus Family (Medical)
The Influenza virus

Bungavirus Family
Genera: Bungavirus: (Medical) California encephalitis, LA Crosse
Phlebovirus: (Medical) Rift Valley Fever
Hantavirus: Puremala is a hemahagin fever virus
Nairvirus: (Veterinary) Nairobi sheep disease
Also many unassigned bungaviruses

Arenavirus Family (Medical)
LCM, Lassa fever virus

Reovirus Family
Genera: Reovirus: a possible human pathogen
Rotavirus: acute gastroenteritis in children
Orbiviruses: (Medical and Veterinary)
Colorado Tick fever, Lebombo (humans) equine encephalosis, blue tongue

Retrovirus Family
Sub-Family:
Oncorivirinal: (Veterinary and Medical) feline leukemia virus, HTLVl and HTLVII

Lentivirinal: (Medical and Veterinary)HIV, feline immunodeficiency virus, equine infections, anemia virus
Spumavirinal

Papovavirus Family
Sub-Family:
Polyomaviruses: (Medical) BKU and JCU viruses
Sub-Family:
Papillomavirus: (Medical) many viral types associated with cancers or malignant progression of papilloma

Adenovirus (Medical)
EX AD7, ARD., O.B.- cause respiratory disease- some adenoviruses such as 275 cause enteritis

Parvovirus Family (Veterinary)
Feline parvovirus: causes feline enteritis
Feline panleucopeniavirus
Canine parvovirus
Porcine parvovirus

Herpesvirus Family
Sub-Family: alphaherpesviridue
Genera: Simplexvirus (Medical)
HSVI, HSVII
Varicellovirus: (Medical and Veterinary) pseudorabies-varicella zoster
Sub-Family: betaherpesviridue
Genera: Cytomegalovirus (Medical)
HCMV
Muromegalovirus
Sub-Family: Gammaherpesviridue
Lymphocryptovirus (Medical)
EBV- (Burkitts lympho)
Rhadinovirus

Poxvirus Family
Sub-Family: Chordopoxviridue (Medical and Veterinary)
Genera: Variola (Smallpox)
Vaccinia (Cowpox)
Parapoxivirus- Veterinary
Auipoxvirus- Veterinary
Capripoxvirus
Leporipoxvirus
Suipoxvirus
Sub-Family: Entomopoxviridue

Hepadnavirus Family	Hepatitis B virus
Unclassified	Hepatitis delta virus

Table 3

Bacterial Pathogens

Pathogenic gram-positive cocci include: pneumococcal; staphylococcal; and streptococcal. Pathogenic gram-negative cocci include: meningococcal; and gonococcol.

Pathogenic enteric gram-negative bacilli include: enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigellosis; hemophilus; chancroid; brucellosis; tularemia; yersinia (pasteurella); streptobacillus moniliformis and spirillum; listeria monocytogenes; erysipelothrix rhusiopathiae; diphteria; cholera; anthrax; donovanosis (granuloma inguinale); and bartonellosis.

Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include: syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis.
Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidiodomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis, and dermatophytosis.

Rickettsial infections include rickettsial and rickettsioses.

Examples of mycoplasma and chlamydia infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydia infections.

Pathogenic eukaryotes

Pathogenic protozoans and helminths and infections thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

Claims

1. A composition comprising at least one nucleic acid molecule that encodes VEGF operably linked to regulatory elements, and at least one nucleic acid molecule that encodes at least one immunomodulating protein operably linked to regulatory elements, wherein said immunomodulating protein is selected from the group consisting of GM-CSF, IL-12, IL-15, IL-2, B7.1, B7.2, MCP-1, MIP-1α, MIP-1β, IL-8, RANTES, L-selectin, P-selectin, E-selectin, CD34, GlyCAM-1, MadCAM-1, LFA-1, VLA-1, Mac-1, p150.95, PECAM, ICAM-1, ICAM-2, ICAM-3, CD2, LFA-3, M-CSF, G-CSF, IL-4, mutant forms of IL-18, CD40, CD40L, vascular growth factor, IL-7, nerve growth factor, Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6 and Caspase ICE.

2. The composition of claim 1 wherein the immunomodulating protein is selected from the group consisting of GM-CSF, IL-12, IL-15, IL-2, B7.1, B7.2, CD40L, MCP-1, MIP-1α, MIP-1β, IL-8, RANTES, L-selectin, P-selectin, E-selectin, CD34, GlyCAM-1, MadCAM-1, LFA-1, VLA-1, Mac-1, p150.95, PECAM, ICAM-1, ICAM-2, ICAM-3, CD2, LFA-3, M-CSF, G-CSF, IL-4, mutant forms of IL-18, CD40, vascular growth factor, IL-7, and nerve growth factor.

3. The composition of claim 1 wherein the immunomodulating protein is selected from the group consisting of GM-CSF, IL-12, IL-15, and IL-2, B7.1, B7.2 and CD40L.

4. The composition of claim 3 wherein the immunomodulating protein is GM-CSF.

5. The composition of claim 1 which additionally comprises at least one nucleic acid that encodes at least one immunogen operably linked to regulatory elements.

6. The composition of claim 5 wherein the immunogen is a protein selected from the group consisting of a pathogen antigen, a cancer-associated antigen and an antigen linked to cells associated with autoimmune diseases.

7. The composition of claim 1 wherein the nucleic acid molecule is selected from the group consisting of a plasmid and a vector.

8. A cell comprising the nucleic acid molecule of claim 7.

9. The composition of claim 2 wherein the nucleic acid molecule is selected from the group consisting of a plasmid and a vector.

10. A cell comprising the nucleic acid molecule of claim 9.

11. The cell of claim 10 which is a pathogenic cell.

12. The cell of claim 11 wherein the pathogenic cell is attenuated.

13. The composition of claim 1 wherein the immunomodulating protein is selected from the group consisting of FasL, TNF-α, and TNF-β.

14. The composition of claim 1 wherein the immunomodulating protein is selected from the group consisting of Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6, and Caspase ICE.

15. The composition of claim 14 which additionally comprises a nucleotide sequence encoding a cell-specific ligand operably linked to regulatory elements.

16. The composition of claim 15 wherein the cell-specific ligand is selected from the group consisting of an antibody and an antigen.

17. A pharmaceutical composition comprising the composition of claim 1.

18. A method for enhancing immune response in an individual comprising administering to the individual the composition of claim 2.

19. A method for enhancing immune response in an individual comprising administering to the individual the composition of claim 3.

20. A method for inducing an immune response in an individual against an immunogen comprising administering to the individual the composition of claim 5.

21. A recombinant vaccine comprising the composition of claim 5.

22. A method for suppressing immune response in an individual comprising administering to the individual the composition of claim 14.

23. A method for inducing apoptosis in a target cell population in an individual comprising administering to the individual the composition of claim 15, wherein the cell specific ligand is specific for the target cell population.

24. A vector for gene therapy, comprising the nucleotide acid molecule of claim 2 in a vector suitable for the transformation of mammalian cells.

25. A composition comprising

a) VEGF and/or at least one nucleic acid molecule that encodes VEGF operably linked to regulatory elements and

b) at least one immunomodulating protein and/or at least one nucleic acid molecule that encodes at least one immunomodulating protein operably linked to regulatory elements,

wherein said immunomodulating protein is selected from the group consisting of: GM-CSF, IL-12, IL-15, IL-2, B7.1, B7.2, MCP-1, MIP-1α, MIP-1β, IL-8, RANTES, L-selectin, P-selectin, E-selectin, CD34, GlyCAM-1, MadCAM-1, LFA-1, VLA-1, Mac-1, p150.95, PECAM, ICAM-1, ICAM-2, ICAM-3, CD2, LFA-3, M-CSF, G-CSF, IL-4,

mutant forms of IL-18, CD40, CD40L, vascular growth factor, IL-7, nerve growth factor, Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6, and Caspase Ice.

26. The composition of claim 1, wherein the immunomodulating protein is selected from the group consisting of GM-CSF, IL-12, IL-15, IL-2, B7.1, B7.2, CD40L, MCP-1, MIP-1α, MIP-1β, IL-8, RANTES, L-selectin, P-selectin, E-selectin, CD34, GlyCAM-1, MadCAM-1, LFA-1, VLA-1, Mac-1, p150.95, PECAM, ICAM-1, ICAM-2, ICAM-3, CD2, LFA-3, M-CSF, G-CSF, IL-4, mutant forms of IL-18, CD40, vascular growth factor, IL-7, and nerve growth factor.

27. The composition of claim 25 wherein the immunomodulating protein is selected from the group consisting of GM-CSF, IL-12, IL-15 and IL-2, B7.1, B7.2 and CD40L.

28. The composition of claim 27 wherein the immunomodulating protein is GM-CSF.

29. The composition of claim 25 which additionally comprises an immunogenic protein and/or at least one nucleic acid that encodes at least one immunogenic protein operably linked to regulatory elements.

30. The composition of claim 29 wherein the immunogenic protein is a protein selected from the group consisting of a pathogen antigen, a cancer-associated antigen and an antigen linked to cells associated with autoimmune diseases.

31. The composition of claim 25 wherein the immunomodulating protein is selected from the group consisting of fasL, TNF-α, and TNF-β.

32. The composition of claim 25 wherein the immunomodulating protein is selected from the group consisting of Fas, TNF receptor, Flt, Apo-1, p55 WSL-1, DR3, TRAMP,

Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6, and Caspase ICE.

33. The composition of claim 32 which additionally comprises a nucleotide sequence encoding a cell-specific ligand operably linked to regulatory elements.

34. The composition of claim 33 wherein the cell-specific ligand is selected from the group consisting of an antibody and an antigen.

35. A pharmaceutical composition comprising the composition of claim 25.

36. A method for enhancing immune response in an individual comprising administering to the individual the composition of claim 26.

37. A method for enhancing immune response in an individual comprising of administering to the individual the composition of claim 27.

38. A method for inducing an immune response in an individual against an immunogenic protein comprising administering to the individual the composition of claim 29.

39. A method for suppressing immune response in an individual comprising administering to the individual the composition of claim 32.

40. A method for inducing apoptosis in a target cell population in an individual compromising administering to the individual the composition of claim 33 wherein the cell-specific ligand is specific for the target cell population.

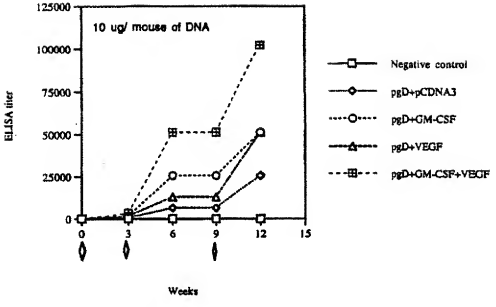
10 ug/ mouse of DNA
ELISA titer
125000
100000
75000
50000
25000
0
0
3
6
9
12
15
Weeks
Negative control
pgD+pCDNA3
pgD+GM-CSF
pgD+VEGF
pgD+GM-CSF+VEGF

FIGURE 1

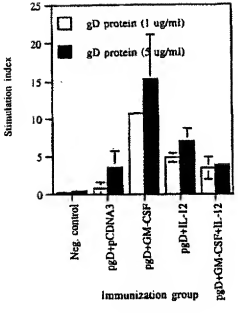

FIGURE 2A

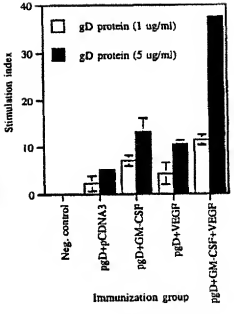

FIGURE 2B

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(54) Title: VACCINES, IMMUNOTHERAPEUTICS AND METHODS OF USING THE SAME

(57) Abstract: Compositions for and methods of enhancing, suppressing or otherwise modulating immune responses are disclosed. Improved vaccines which include a nucleotide sequence that encodes VEGF and an immunomodulating protein, both operably linked to regulatory elements are disclosed. The improved vaccines include DNA vaccines, recombinant vaccines for delivering foreign antigen and live attenuated vaccines. Methods of immunizing individuals are disclosed. Compositions for and methods of treating individuals with autoimmune diseases are disclosed.

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A. CLASSIFICATION OF SUBJECT MATTER
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US CL : 424/184.1; 435/320.1; 514/44; 530/350; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/184.1, 435/320.1, 514/44, 530/350, 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, BIOSIS, CAPLUS, MEDLINE, CANCERLET, SCISEARCH, EMABSE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,090,790 A (ERIKSSON) 18 JULY 2000 (18.07.2000) See particularly claims 1 and 2.	1-4, 7-10, 13-15, 17, 24, 25, 26, 27, 28, 31-33, 35
Y	US 5,919,459 A (NACY ET AL) 06 JULY 1999 (06.07.1999), See particularly abstract, column 1, lines 20-48, example 1.	1-40
Y	US 5,738,852 A (ROBINSON ET AL) 14 APRIL 1998 (14.04.1998) See abstract, columns 9 and 10.	1-40
Y	US 6,197,743 A (FALLER) 06 MARCH 2001 (6.03.2001) see particularly column 19, lines 45-59).	1-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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